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Characterisation of fibropapillomatosis tumour growth profiles in green sea turtles (*Chelonia mydas*)

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Background

Wild sea turtle populations are currently listed as threatened and endangered as a result of both natural and anthropogenic factors (Jones *et al.* 2016; Duffy *et al.* 2018; IUCN 2018). These include predation, disease, starvation, pollution, fisheries interaction and habitat degradation. Many natural threats including disease outbreaks have been exacerbated by human interaction (Dos Santos *et al.* 2010; Jones *et al.* 2016; Whilde *et al.* 2017). One disease of increasing threat to marine turtle populations worldwide is fibropapillomatosis (FP), a virulent cancer (Fig. 1), thought to be triggered by a chelonian-specific herpes virus, ChHV5 (Herbst *et al.* 1995; Jones *et al.* 2016; Work *et al.* 2017; Morrison *et al.* 2018).

First reported in the scientific literature in 1938, FP prevalence was as low as 1.5% in the Florida Keys at that time (Smith & Coates 1938). Eyewitness sources suggest the first cases occurred as early as the late 1800s (Cruz 1985). Fibropapillomatosis did not reach epizootic proportions until the 1980s when a combination of increased coastal development and human-induced climate change began to significantly degrade juvenile sea turtle habitats. Presenting as single or several benign fibroepithelial cutaneous

lesions (Jones *et al.* 2016), the disease significantly affects turtle survivorship once the tumours are of significant size in locations inhibiting vision, feeding, internal organ function and locomotive ability. The disease progressed to a panzootic in the 1990s with outbreaks in the eastern Pacific, Hawaiian Islands, Indonesia and Australia (Duarte *et al.* 2012; Page-Karjian *et al.* 2014; Hargrove *et al.* 2016; Jones *et al.* 2016). Currently, there are a number of FP visual scoring systems including the fibropapillomatosis index (FPI) (Rossi *et al.* 2016) and a four-category size classification which ranks the tumour burden from 0 to 3 (Work & Balazs 1999).

The number of turtles stranding with FP in Florida has exploded in recent years, with over 250 entering Florida rehabilitation facilities in 2017. This trend is likely to continue into the future (Foley 2015; Hargrove *et al.* 2016; Duffy *et al.* 2018). Reported in all seven marine turtle species, FP most severely impacts green sea turtles (*Chelonia mydas*). Evidence suggests that its geographic range is spreading to more northern latitudes where FP was never previously recorded. The disease is now undermining conservation efforts across the globe (Duarte *et al.* 2012; Page-Karjian *et al.* 2014; Hargrove *et al.* 2016; Jones *et al.* 2016; Duffy *et al.* 2018).

Currently, the only commonly applied treatment for FP is surgery, which is expensive, restricted to only a small number of turtle hospitals and 60% of the time results in tumour regrowth post-surgery (Page-Karjian *et al.* 2014; Whilde *et al.* 2017). While the occurrence of FP tumours and post-surgical regrowth has been recorded previously, the specific growth rates of tumours pre- and post-surgery have never been assessed in detail. Such data are vital for establishing a growth baseline to better understand the disease and factors that may be accelerating its growth. Additionally, tumour growth baselines are crucial when assessing the effectiveness of targeted therapeutics. Once baseline FP tumour growth rates are established, these can be used to compare the rate of tumour growth and regrowth in patients subjected to various candidate drug treatments in order to identify the most effective course of treatment for FP-afflicted patients. Innovative treatment approaches are vital to help maintain healthy marine populations until the chronic underlying causes of these diseases can be addressed and preventative measures can be enforced (Duffy *et al.* 2018).

Objectives

This paper documents the growth and post-surgical regrowth rates of FP tumours in four *C. mydas* patients at the University of Florida's Whitney Sea Turtle Hospital. These data will indicate which tumour locations are more susceptible to accelerated tumour regrowth. The results will also be useful as baseline information for future drug treatment studies, as well as suggesting further beneficial refinements to tumour growth profiling techniques.

Method

Sample population: This study used existing images, taken as part of the rehabilitative care of four juvenile *C. mydas* patients admitted to the Whitney Sea Turtle Hospital between September 2016 and April 2017. Images were taken using an Olympus Tough TG-4 approximately 30cm from each lesion, with a 25cm scale bar for accurate pixel comparison. All four patients were found stranded along the east coast of Florida between Anastasia State Park (north, 29.9083°N, 81.2837°W) and Ponce Inlet (south, 29.0779°N, 80.9211°W), displaying fibropapilloma-like tumours. Each patient received medical care devised to best suit their particular circumstances; thus the number of surgeries and duration of tumour growth analysis varies for each turtle. The outcome of each patient differs, with some determined healthy enough for release and others requiring euthanasia and necropsy. Tumour removal surgeries involved the use of a CO₂ laser to excise tumours and cauterize surgical incision sites.

Table 1. Carapace length, weight, origin, condition on arrival and FP tumour scores of green sea turtles (*C. mydas*) observed during this study at the Whitney Laboratory for Marine Bioscience Sea Turtle Hospital.

Patient ID	Straight carapace length (upon admittance) cm	Weight (upon admittance) kg	Origin	Condition on arrival	FP tumour score (FPI) (Rossi <i>et al.</i> 2016)
Chrystal	29.2	2.8	Volusia FL	Normal body condition, FP	>66.5
Tamatoa	33.6	4.6	St. Johns FL	Normal body condition, FP	22.1
Pons	42.8	7.5	Volusia FL	Emaciated, FP	>6.5
Remi	35.7	3.8	Volusia FL	Emaciated, FP	>205.6

Image analysis: ImageJ is an image processing programme designed for scientific multidimensional images (<https://imagej.nih.gov/ij>). Image analysis using this computer software allows accurate measurements of tumour two-dimensional surface area. It should be noted that the surface measurements used in this study serve as a proxy for overall tumour growth. In future studies, physical measurements of the three-dimensional tumour structures will allow a more in-depth direct assessment of tumour growth dynamics. However, here we evaluated the use of ImageJ software to track tumour growth. Existing historical FP patient datasets primarily only contain visual records of tumour growth, so, if found to be an applicable approach, two-dimensional surface

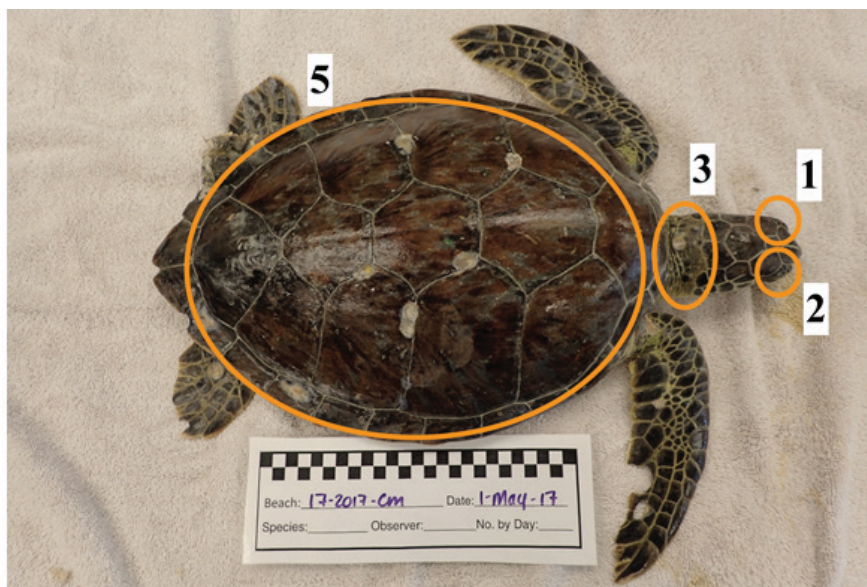


Fig. 1. Location of designated FP tumour 'clusters' on green sea turtle anatomy (Patient 4: Remi).

area measurement would then be retroactively applicable to a large cohort of patient data. Each tumour is assigned to a 'cluster' to enable analysis of tumour growth in designated locations on the turtle anatomy (Fig. 1 and Table 2). Each cluster was photographed by a member of the Whitney Sea Turtle Hospital veterinary team on arrival, at each check-up and after each surgery. These images were analysed in ImageJ in order to plot the growth (surface area) and post-surgical regrowth of each cluster.

Table 2. Body location and abbreviations of tumour clusters used in this study.

Location designated as tumour clusters	Cluster abbreviation	Figure 1 Cluster location number
Left Eye	LEy	1
Right Eye	REy	2
Dorsal Neck	DNk	3
Ventral Neck	VNk	4
Carapace	Carapace	5
Plastron	Plastron	6
Left Front Flipper Base	LFFBa	7
Left Front Flipper Along	LFFAI	8
Right Front Flipper Base	RFFBa	9
Right Front Flipper Along	RFFAI	10
Left Rear Flipper Base	LRFBa	11
Left Rear Flipper Along	LRFAI	12
Right Rear Flipper Base	RRFBa	13
Right Rear Flipper Along	RRFAI	14
Tail	Tail	15

Results

Patient summaries

PATIENT 1: CHRYSTAL

Chrystal was first photographed at the Whitney Sea Turtle Hospital on January 12th 2017 (Fig. 2A). She underwent three surgeries to mitigate the severity of her fibropapillomatosis burden. Photographs were taken after each of Chrystal's surgeries on the following dates:

- ❑ February 8th: removal of tumours from Left Eye (LEy), Right Eye (REy), Right Rear Flipper Base (RRFBa) and Left Front Flipper Base (LFFBa).
- ❑ May 1st: removal of tumours from Right Front Flipper Base (RFFBa) and Right Front Flipper Along (RFFAI).
- ❑ June 13th: removal of tumours from LEy and REy (for the second time) and Dorsal Neck (DNk).

The most persistent tumour regrowth was seen on Chrystal's eyes (Fig. 2B & Table 3). Limited regrowth was seen on the RFFBa and RFFAI (Fig. 2B). No regrowth was seen on the LFFBa. All clusters left untreated by surgery showed continuous steady growth. Due to continual tumour regrowth and the diagnosis of internal lung tumours and impacts on quality of life, Chrystal was euthanised on August 9th 2017, after 210 days in rehab, with a thorough necropsy providing evidence of significant internal FP tumour growth – in particular, a large golf ball-sized tumour located within the shoulder of the RFFBa. The necropsy determined that Chrystal was a female.

A



B

Patient 1: Chrystal

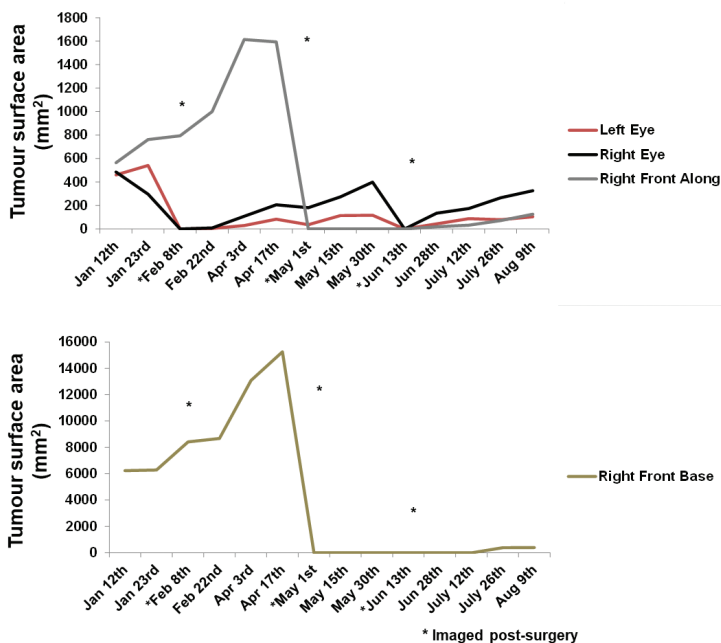


Fig. 2. Fibropapillomatosis in a green turtle (Patient 1: Chrystal).

(A) Chrystal's initial intake photograph upon admittance to the hospital.

(B) Growth profile of selected FP tumour clusters on Chrystal.

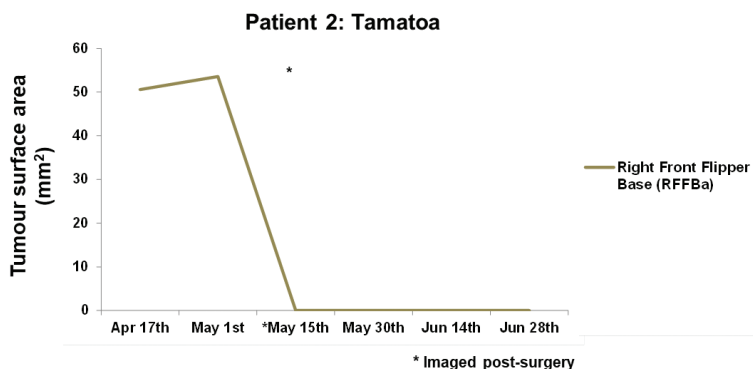
A**B**

Fig. 3. Patient 2: Tamatoa. **(A)** Tamatoa's initial intake photograph upon admittance to the hospital. Tamatoa was found stranded by the Salt Run fish cleaning table in Anastasia State Park, where he was habitually fed by local fishermen. **(B)** Growth profile of the surface area of Tamatoa's RFFBa cluster, obstructed in (A) due to placement of flipper.

PATIENT 2: TAMATOA

Tamatoa was admitted to the Whitney Sea Turtle Hospital on April 17th 2017 (Fig. 3A), after stranding at Anastasia State Park, Florida.

- ❑ Tamatoa underwent one surgery on May 15th 2017 to remove tumours from its RFFBa, LFFBa and RFBa.

Tamatoa showed no signs of tumour regrowth post-surgery (Fig. 3B & Table 3). Showing good health and no indication of regrowth, Tamatoa was released on July 13th 2017, after 88 days in rehab. Tamatoa was subsequently found re-stranded and alive on August 27th 2017 at Anastasia State Park as a result of being caught in a cast-net. There was no indication of FP tumours.

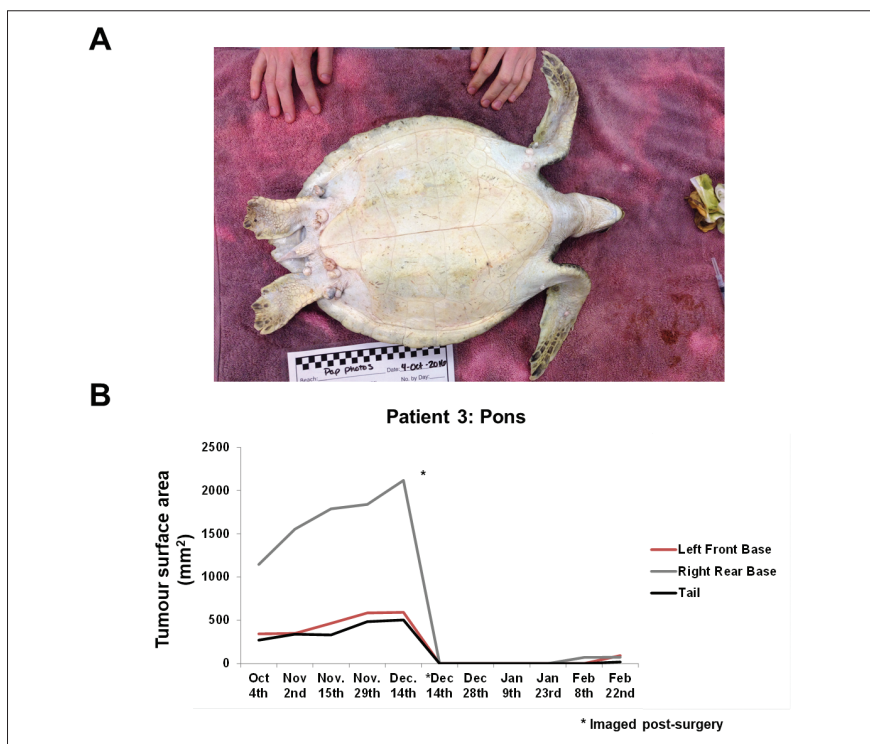


Fig. 4. Patient 3: Pons.

(A) Pons's initial intake photograph upon admittance to the hospital. Found floating in Ponce Inlet, Volusia County, with monofilament tangled around the right rear flipper base.

(B) Growth profile of selected FP tumour clusters on Pons.

PATIENT 3: PONS

Pons was reported as stranded to the Whitney Sea Turtle Hospital on September 25th 2016 and was collected from the water at Ponce Inlet, Florida. Pons was first photographed at the Whitney Sea Turtle Hospital on October 4th 2016 (Fig. 4A). Pons was thin, with a healed propeller wound to the first vertebral and left costal, and minimal algae and barnacles on the carapace.

- Pons underwent one surgery on December 14th 2017 to remove tumours from its LFFBa, RFFBa, LRFBa, RRFBa and Tail.

Pons showed the start of very minor regrowth on the LFFBa, RRFBa and Tail (Fig. 4B). No regrowth was seen on the RFFBa or LRFBa. Showing good health and no further regrowth, Pons was released on March 16th 2017, after 173 days in rehab. Pons was subsequently caught and released by a fisherman on March 28th 2017; however, there was no indication of FP tumours, suggesting that the beginnings of regrowth regressed while in the wild.

A



B

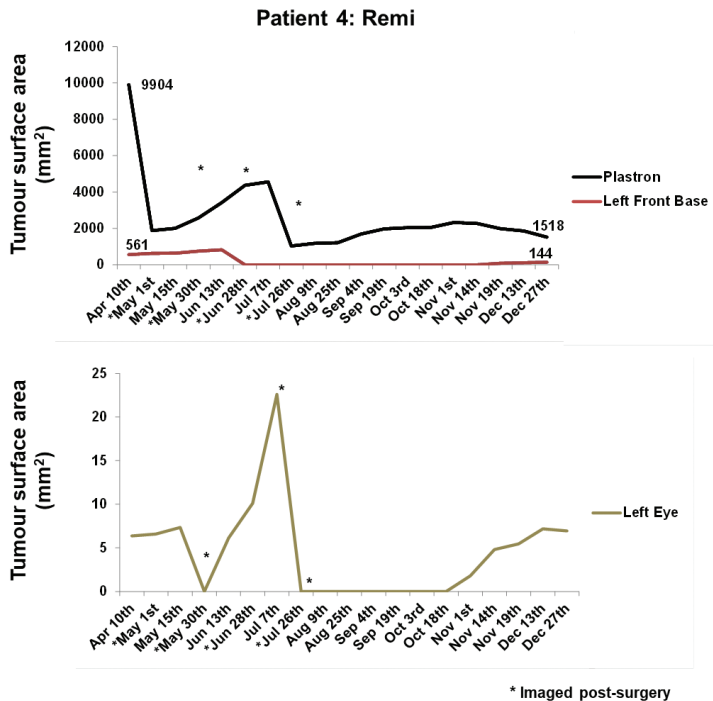


Fig. 5. Patient 4: Remi.

(A) Remi's initial intake photograph upon admittance to the hospital. Remi was found and collected from the inshore waters of South Daytona Beach Canal off the Halifax River, underweight and lacking in energy.

(B) Growth profile of selected FP tumour clusters on Remi.

PATIENT 4: REMI

Remi was reported as stranded to the Whitney Sea Turtle Hospital on April 10th 2017 and was collected from the inshore waters of Halifax River, Florida. Remi was first photographed at the Whitney Sea Turtle Hospital on April 10th 2017 (Fig. 5A). Remi was thin and lethargic with mud and algae covering his carapace. The patient underwent four surgeries to mitigate the severity of its FP burden. Photographs were taken after each of Remi's surgeries on the following dates in 2017:

- ❑ May 1st: removal of tumours from Carapace, RFFAI, RFFBa and Plastron.
- ❑ May 30th: removal of tumours from LEy.
- ❑ June 28th: removal of tumours from LFFAI, LFFBa, LRFBa and LRFAL.
- ❑ July 26th: removal of tumours from RRFBa, RRFAI, LEy (for the second time), Ventral Neck (VNk) and Plastron (for the second time).

The most persistent tumour regrowth was seen on Remi's left eye (LEy) as well as the Plastron (Fig. 5B). Minor regrowth was seen on the LFFBa (Fig. 5B). No regrowth was seen on the Carapace, VNk, RFFBa, RFFAI, LFFAI, LRFBa, LRFAL, RRFBa and RRFAI. Due to its multiple surgeries and continued regrowth Remi remained in the care of the Whitney Sea Turtle Hospital for a total of 344 days, but was successfully released on 20th March 2018.

Tumour growth rates by individual and cluster locations

We assessed the growth rates of all tumour clusters, examining whether there was any correlation in cluster location and the pre-surgery growth rate, across all four patients. Generally, plastron tumours and those at the base of the flippers tended to grow at a faster rate than other locations (Fig. 6A), although it should be noted that these clusters also tended to have the largest tumour burdens (Table 3). Regrowth data were not available for some of Chrystal's FP clusters, as surgery took place too soon after the first photograph to have a second image available (Table 3).

We next compared the predicted doubling time of each tumour (start size/pre-surgery growth rate). Doubling time represents the hypothetical time it would take (in days) for a tumour to double its original size (size at commencement of measuring), assuming it continued to grow at its pre-surgery rate. While there was a slight trend for larger tumours to require a longer doubling time (Fig. 6B), despite their more rapid growth rate (Table 3), there was no strong correlation between the size of a tumour and its predicted doubling time (linear correlation, $R^2 = 0.01296$).

In addition to characterising tumour growth rates as a baseline for future studies, we also investigated whether there was any prognostic value in the pre-surgery growth rates in terms of predicting patient outcome or the occurrence of post-surgery tumour regrowth. However, the average tumour regrowth across all clusters of an individual turtle were not predictive of rehabilitation outcome (Fig. 6C).

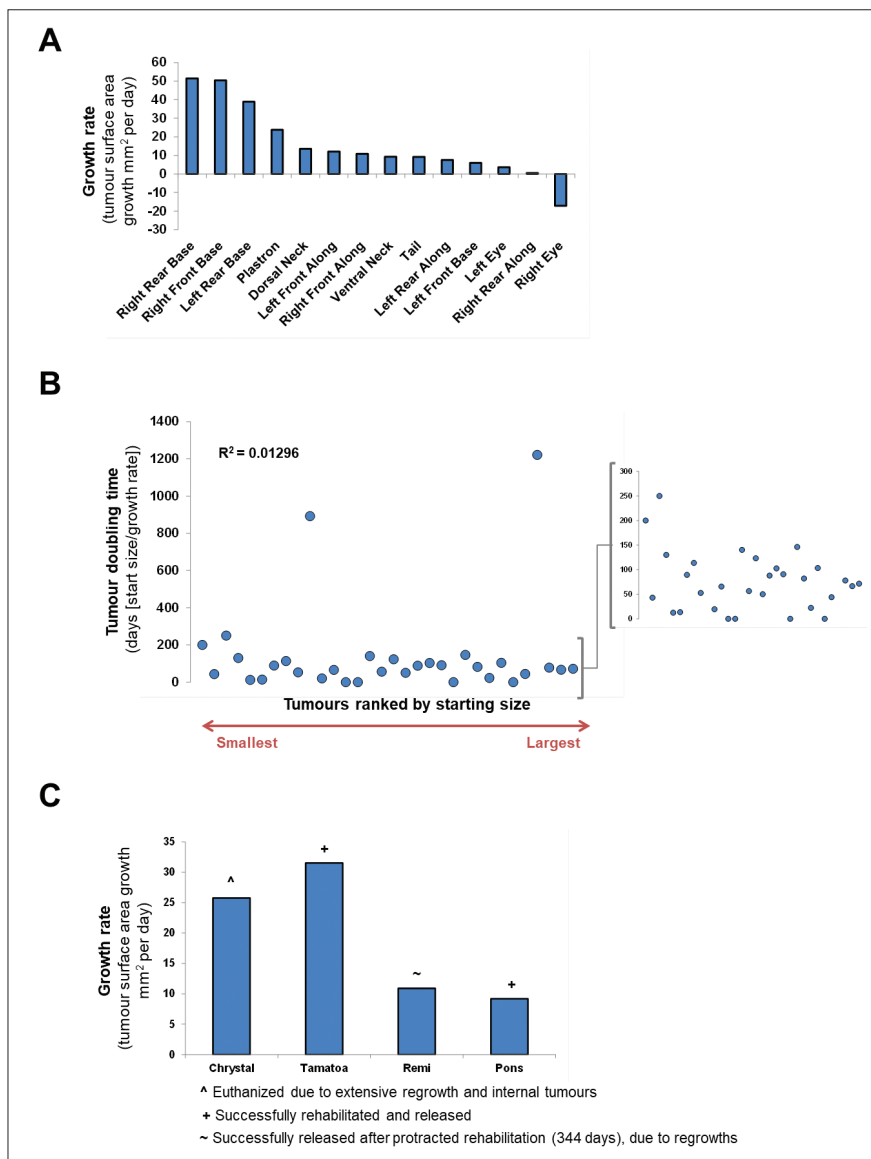
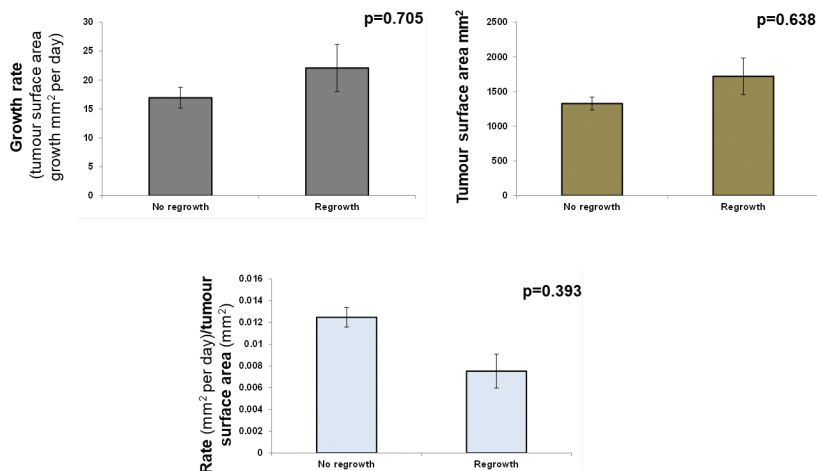


Fig. 6. Analysis of tumour growth rates.

(A) Average pre-surgery tumour growth rates combined by cluster, across all four patients.
(B) Predicted tumour doubling time by tumour starting size. Doubling time represents the hypothetical time it would take (in days) for a tumour to double its original size, assuming it had been allowed to continue to grow and maintained its pre-surgery growth rate.
(C) Average pre-surgery tumour growth rates combined by individual patient, related to rehabilitation outcome.

A



B

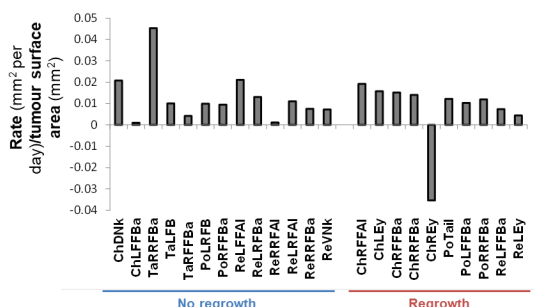


Fig. 7. Correlation of pre-surgery growth dynamics with the occurrence of post-surgical FP tumour regrowth.

(A) Average pre-surgery tumour growth rates (left), tumour starting size (right) and growth rate/starting size (bottom), across all patients and clusters (for which regrowth information existed) grouped according to the occurrence of post-surgical tumour regrowth. Error bars denote standard error.

(B) Tumour growth rate/starting size across patient clusters, grouped according to the occurrence of post-surgical tumour regrowth.

Next we examined whether any of the tumours' growth characteristics (starting size, growth rate or growth rate/starting size) were predictive of the occurrence of post-surgery regrowth (Fig. 7A). Interestingly, for those tumour clusters with growth rate data available, the pre-surgery growth rate did not clearly indicate whether a tumour would regrow post-surgery (Fig. 7A, B).

Neither starting size (t-test, $p = 0.6382$, d.f. = 21), growth rate (t-test, $p = 0.7050$, d.f. = 21) or growth rate/starting size (t-test, $p = 0.3931$, d.f. = 21) were significantly different between clusters exhibiting regrowth and those having no post-surgery regrowth.

It should be noted that the negative growth rate for Chrystal's REy cluster was as a result of initial inflammation in that tumour being reduced upon commencement of rehabilitative care. The reduction in size was not due to genuine natural tumour regression. This inflammation complication also tallies with the occurrence of post-surgical tumour regrowth in this eye. However, even if this cluster is excluded from the analysis there remains no significant difference between the no regrowth and regrowth clusters (t-test for growth rate $p = 0.4925$, d.f. = 20, t-test for rate/starting size, $p = 0.9634$, d.f. = 20). Given this lack of significance we can rule out the use of these growth characteristics as prognostic markers of tumour regrowth. However, reassessing these features with a larger sample size would of course be highly desirable.

Discussion

Patients 2 and 3 received one surgery only as their symptoms were far less advanced than Patients 1 and 4. As their FP was less advanced, they showed minor or no regrowth and were released back into their wild populations. Coincidentally, both patients were caught alive and released by fishermen approximately one month later; however, there was no evidence of tumour regrowth. As the patients were tagged, the fishermen took photos prior to release to send to the hospital so as to keep track of patient health and location.

Patients 1 and 4 displayed far more severe FP symptoms. Despite multiple rounds of surgery, persistent tumour regrowth was a recurring problem. The most susceptible sites requiring multiple surgeries were the eyes and the plastron. Therefore, these sites should receive special focus in any future adjunct post-surgery drug treatment trials. Due to the severity of their symptoms, Patient 1 required euthanasia and Patient 4 remained in rehabilitation almost one year after its initial stranding. Patient 1 displayed continuous tumour regrowth and poor health. Her necropsy subsequently showed extensive growth of internal FP tumours.

The two smallest patients, Chrystal and Remi (Table 1), were those most affected by post-surgical tumour regrowth. Our observation supports the findings made by Page-Karjian *et al.* (2014) that smaller turtles (straight carapace lengths 30-35cm) were the most susceptible to FP tumour development while in rehabilitation. Page-Karjian *et al.* (2014) suggested that turtles are more likely to develop FP tumours during warmer rehabilitation months (April-September), an observation also postulated elsewhere (Cruz 1985; Herbst *et al.* 1995; Jones *et al.* 2016; Duffy *et al.* 2018). This association

Table 3. Pre-surgery fibropapillomatosis tumour growth characteristics and post-surgery regrowth rates.

Patient	Cluster	Start Size (mm ²)	Growth Rate Prior To Surgical Removal (mm ² per day)	Growth Rate/ Start Size	Re-Growth Y/N	Growth Period (Days)
1. Chrystal	Left Eye	460	7.0	0.016	Y	11
	Right Eye	485	-17.0	-0.035	Y	11
	Dorsal Neck	651	13.0	0.021	N	138
	Ventral Neck	1476	18.0	0.012	N/A	209
	Plastron	447	23.0	0.053	N/A	209
	Left Front Base	3662	3.0	0.001	N	11
	Left Front Along	202	15.0	0.076	N/A	209
	Right Front Base	6234	94.0	0.015	Y	95
	Right Front Along	564	10.0	0.019	Y	95
	Left Rear Base	2111	48.0	0.023	N/A	209
	Left Rear Along	43	1.0	0.024	N/A	209
	Right Rear Base	7134	100.0	0.014	Y	11
	Tail	185	15.0	0.081	N/A	209
2. Tamatoa	Left Front Base	1232	12.0	0.010	N	14
	Right Front Base	50	0.2	0.004	N	14
	Right Rear Base	1806	81.0	0.045	N	14
3. Pons	Left Front Base	341	3.0	0.010	Y	71
	Right Front Base	616	5.0	0.010	N	71
	Left Rear Base	1964	19.0	0.010	N	71
	Right Rear Base	1143	13.0	0.012	Y	71
	Tail	268	3.0	0.012	Y	71
4. Remi	Left Eye	6	0.03	0.004	Y	35
	Ventral Neck	78	0.6	0.007	N	88
	Carapace	1981	N/A	N/A	N	21
	Plastron	9903	N/A	N/A	Y	21
	Left Front Base	561	4.0	0.007	Y	64
	Left Front Along	421	8.0	0.021	N	64
	Right Front Base	1282	N/A	N/A	N	21
	Right Front Along	528	N/A	N/A	N	21
	Left Rear Base	3738	48.0	0.013	N	64
	Left Rear Along	1270	14.0	0.011	N	64
	Right Rear Base	1316	9.0	0.008	N	88
	Right Rear Along	446	0.5	0.001	N	88

between warmer months and higher regrowth rates was also apparent with both Patients 1 and 4 in this study. The most commonly FP-afflicted body locations are stated to be the R/LFFBa, R/LFFAI, Tail, Plastron and R/LEy (Page-Karjian *et al.* 2014). Again, the patients in this study support this finding. The presence of ocular tumours can be a good indication of the outcome of the

patient, as turtles without FP lesions on the eye are eight times more likely to survive (Page-Karjian *et al.* 2014). The results from this study also support this suggestion, as those turtles without eye tumours were able to be released, while those with eye tumours required euthanasia or prolonged rehabilitation time and medical care. In previous studies, the number of tumour removal surgeries has not significantly related to patient outcome (Page-Karjian *et al.* 2014). However, in this study a greater number of surgeries did coincide with worse outcomes (euthanasia, extended rehabilitation time). In previous studies, 38.5% of turtles experienced post-surgical regrowth within an average of 36 days (Page-Karjian *et al.* 2014). In line with this, in our current study 50% of turtles experienced FP regrowth within 40 days. Of the 33 tumour clusters surgically removed from these 4 patients, one third resulted in tumour regrowth. This is slightly less than the 60% tumour regrowth observed in previous studies (Page-Karjian *et al.* 2014).

The growth measurement protocol adopted in this study can be further improved for future research in order to increase its accuracy and the level of detailed analysis which is possible. Photos could be taken at a fixed distance and at a consistent angle for each tumour cluster, and physical measurements using callipers could be used to record accurate tumour dimensions. While providing more precise data, these physical methods, however, are considerably more time-consuming. Thus they are less likely to be broadly adopted across many rehabilitation facilities, which are generally time- and resource-limited.

Interestingly, this study suggests that the aggressiveness of the pre-surgery growth rates do not have a bearing on the occurrence of post-surgery tumour regrowth rates. Therefore, regrowth may be driven predominantly by other factors such as inherent genetic/viral features of each tumour, or tumour cells/tumour stem cells remaining in the body site post-surgery (deeper surgical margins may alleviate this, although these are not possible in some locations such as the eye). In line with the potential drivers of FP regrowth, we have recently shown that adjunct post-surgery treatment with the anti-cancer drug fluorouracil can help to dramatically reduce FP eye tumour regrowth (Duffy *et al.* 2018).

The analysis of tumour growth in these four patients provides a useful baseline with which to compare FP tumour growth rates in *C. mydas* given novel FP treatments. Future studies can compare this baseline with growth post-candidate drug treatment. Additionally, these data will provide useful baseline information for studies investigating the effect of potential environmental aggravators of FP tumour growth, such as UV exposure and pollutant exposure (Keller *et al.* 2014; Jones *et al.* 2016; Duffy *et al.* 2018). If growth and regrowth rates can be reduced from the baseline rates indicated in this study, it could eliminate the need for multiple rounds of

surgery, consequently decreasing the stress on the patient and increasing their chances of successful re-introduction to their wild population. At a time when anthropogenic factors are accelerating disease emergence and species extinction (Whilde *et al.* 2017), it is vital to not only expand our scientific understanding of the mechanics of diseases such as fibropapillomatosis, but to use the knowledge gained to improve the care and recovery of endangered animal populations.

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